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PII: S0167-7012(18)30506-2
DOI: doi:10.1016/j.mimet.2018.08.008
Reference: MIMET 5454

To appear in: *Journal of Microbiological Methods*

Received date: 22 June 2018
Revised date: 13 August 2018
Accepted date: 15 August 2018

Please cite this article as: Mathieu Clément, Thomas Büdel, Odette J. Bernasconi, Luigi Principe, Vincent Perreten, Francesco Luzzaro, Andrea Endimiani , The EDTA-based disk-combination tests are unreliable for the detection of MCR-mediated colistin-resistance in Enterobacteriaceae. Mimet (2018), doi:10.1016/j.mimet.2018.08.008

The EDTA-based Disk-Combination Tests Are Unreliable for the Detection of *MCR*-Mediated Colistin-Resistance in *Enterobacteriaceae*

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Running title: EDTA-based disk-combination tests to detect mcr producers

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ABSTRACT

We evaluated several EDTA-based combined-disk tests to detect 25 mcr producers among 48 *Enterobacteriaceae*. Colistin disks plus EDTA (292/584 µg) on MH and CAMH agar were used. Results were positive if with chelator there was an inhibition zone increase ≥3mm compared to colistin alone. All tests resulted unreliable (sensitivity ≤68%).

Keywords: *mcr-1*, colistin, EDTA, *Enterobacteriaceae*, disk, phenotype

Polymyxins are considered the last antibiotic options to treat infections due to extensively drug resistant Gram-negative bacteria. Consequently, the recent pandemic emergence of the plasmid-mediated *mcr* colistin (COL) resistance genes among *Enterobacteriaceae* (especially *Escherichia coli*) represents a serious concern [1-4].

Several molecular methods have been designed to detect *mcr*-based COL resistance, but most of them are expensive, do not simultaneously detect all *mcr* variants so far reported, and cannot identify new emerging *mcr*-like genes [5-10]. Therefore, phenotypic methods are still essential for clinical laboratories that are unable to perform molecular tests, but also to screen large collections of COL-resistant (COL-R) strains to identify *mcr*-like genes not yet targeted by the current molecular assays. In this context, some broth microdilution assays and the polymyxin NP test have demonstrated to be accurate in detecting COL resistance [11-16]. However, none of them is able to distinguish the COL-R mcr-producing strains from those expressing chromosomal mechanisms (e.g., amino acid substitutions in the PmrA/B two-component system in *E. coli*) [4]; moreover, such methods are not easily applicable as routine assays in many low- and middle-income countries.

The *mcr* genes encode for zinc-dependent phosphoethanolamine transferases [17, 18], therefore the metallo chelator EDTA may be useful to improve the *in vitro* activity of COL. Recently, Esposito *et al.* indicated that an EDTA-based combined-disk test (EDTA-CDT), performed on Mueller-Hinton (MH) agar plates and using COL disks supplemented with 292 µg of chelator, showed good performance in detecting *mcr-1*-possessing *E. coli* strains [19]. In this work, we evaluated this simple and inexpensive EDTA-CDT against a collection of well-characterized COL-R *Enterobacteriaceae* in order to validate the protocol prior its implementation in our laboratories.

As shown in Table S1, we tested 48 contemporary *Enterobacteriaceae* (of which 44 COL-R [20]) obtained from human, animal, and food chain sources: 31 *E. coli*, 7 *Klebsiella* spp., 3 *Salmonella* spp., 1 *Enterobacter* spp., 1 *Hafnia alvei*, and 5 intrinsically-resistant bacterial species [21, 22]. MICs for COL were obtained implementing the Sensititre GNX2F or EUVSEC microdilution panels (ThermoFisher Scientific). All strains were tested for the presence of *mcr-1* to *mcr-5* using both PCR/sequencing and the new CT103XL microarray [1, 2, 5, 7, 23-25]. For several strains whole genome sequencing, characterization of chromosomal mechanism(s) of COL resistance, and sequence types were also available (Table S1). Overall, 25 out of 44 COL-R strains (21 *E. coli*, 3 *K. pneumoniae*, one *Salmonella* spp.) possessed *mcr* genes: *mcr-1* (n=22), *mcr-2*, *mcr-3*, and *mcr-4* (n=1 each).

CDTs were performed on in-house made 90 mm agar plates according to the CLSI guidelines [26]. Three 10-µg disks of COL (Becton-Dickinson) were placed on MH and cation-adjusted MH (CAMH) agar (Sigma-Aldrich) plates: COL alone, COL plus 10 µl of EDTA (100 mM; equivalent to 292 µg/disk), and COL plus 10 µl of EDTA (200 mM; equivalent to 584 µg/disk). Notably, disks supplemented with chelator were left drying for 15 min before placing them on the corresponding agar. Plates were incubated at 35-36°C for 20 hrs and then visually read by at least two independent experienced operators unaware of the genotype of tested strains. For each strain, CDTs were performed two times in different working days; if results were discordant the assay showing the greater difference in inhibition zone between COL and COL plus EDTA was considered (data depicted in Table S1). Results were categorized as suggested by Esposito *et al.* [19]: i.e., positive if in presence of chelator the strain exhibited ≥3 mm increase in diameter inhibition zone compared to that of COL disk alone. Sensitivity (SN) and specificity (SP) were determined for each assessed CDT test using as gold standard the results of molecular characterization (i.e., presence of a *mcr* gene).

As reported in Table 1, using MH agar and COL disks supplemented with 292 µg of EDTA, we recorded very low SN and SP (overall, 12.0% and 65.2%, respectively). This data was substantially less promising than the one reported by Esposito *et al.* [19], who recorded good SN and SP (96.7% and 89.6%, respectively) after testing 61 *mcr-1*-positive (60 *E. coli* and one *K. pneumoniae*) and 48 *mcr*-negative strains (including 20 *E. coli* and 25 *K. pneumoniae*). However, in that study most strains showed an increase in inhibition zone diameter for the COL disk plus chelator of 3 mm (just above the positivity cut-off), whereas most of our isolates displayed only a 2 mm increase. Therefore, it can be speculated that the different performance of the same CDT observed in the two studies might be linked to the diverse approaches used by the operators in reading the inhibition halo. On the other hand, this phenomenon clearly indicates that this CDT is of difficult implementation in routine clinical laboratories. In this context, we also underline that *i*) for other more standardized CDT assays (e.g., those for detecting ESBL producers) a cut-off ≥5 mm was set [22], and *ii*) disk diffusion yields unreliable results for COL [4, 20, 27].

Nevertheless, aiming to improve the performance of the EDTA-CDT (i.e., increase the inhibition halo for the mcr producers), we also tested the above mentioned 48 strains using COL disks supplemented with 584 µg of chelator (Table S1). Accordingly, the overall SN improved (68.0%), but at the cost of SP (47.8%). For both CDT approaches (COL disk with 292 or 584 µg of EDTA), we also evaluated the implementation of CAMH instead of MH agar plates; however, even under these different conditions both EDTA-CDTs showed unacceptable performances. Finally, we also noted that setting up different cut-offs of interpretation (i.e., ≥2, ≥4, or ≥5 mm increase in inhibition halo for the COL plus EDTA disk) did not significantly improve CDTs accuracy (Table 1).

Based on the overall mentioned results, we conclude that, regardless of the type of agar and concentration of chelator, EDTA-CDTs are unreliable for the detection of mcr-producing

Enterobacteriaceae. This phenomenon can be ascribed to the low diffusion of COL into the agar medium [27], and justify why EUCAST does not recommend the use of disk diffusion for COL susceptibility tests [20]. In this context, we note that recently even a dipicolinic acid (DPA)-based CDT showed insufficient performance in detecting mcr producers [28]. On the other hand, the DPA test was accurate when performed in broth microdilution format [28], suggesting its possible implementation in low-income countries devoid of molecular assays or to detect novel and emerging *mcr*-like genes in large collection of COL-R strains.

ACKNOWLEDGMENTS

We thank Fernanda Pimentel and Regula Tinguely for the technical support. We also thank Drs. P. Nordmann and L. Poirel (University of Fribourg, Fribourg, Switzerland), S. Malhotra-Kumar (University of Antwerp, Wilrijk, Belgium**),** M. Haenni (Université de Lyon - Agence Nationale de Sécurité Sanitaire, Lyon, France), C. Magistrali (Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche, Perugia, Italy), A. Carattoli (Istituto Superiore di Sanità, Roma, Italy) for donating the *mcr*-positive control strains indicated in Table S1 with their corresponding references.

FUNDING

This work was supported by NRP-72, "*National Research Programme, Antimicrobial Resistance*" (Swiss National Science Foundation; grant No. 177378 due to AE). MC is a PhD student (2018-2021) supported by NRP-72. OJB is a PhD student (2015-2018) supported by the Hans Sigrist Foundation (Bern, Switzerland).

DECLARATIONS OF INTEREST

All authors: none to declare

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Table 1. Detection of *mcr*-based colistin (COL) resistance: performance of the combined-disk tests (CDTs) with COL and COL plus EDTA according to the different cut-offs (increase in zone diameter inhibition)

Statistical parameters for the *Enterobacteriaceae* strains (Overall, n=48 of which 25 *mcr*-positive)	**COL / COL + EDTA disks on Mueller-Hinton agar**								**COL / COL + EDTA disks on cation-adjusted Mueller-Hinton agar**							
	COL / COL + 292 µg of EDTA [c] (increase in inhibition halo, mm)				**COL / COL + 584 µg of EDTA [d]** (increase in inhibition halo, mm)				**COL / COL + 292 µg of EDTA [c]** (increase in inhibition halo, mm)				**COL / COL + 584 µg of EDTA [d]** (increase in inhibition halo, mm)			
	≥2	**≥3**	**≥4**	**≥5**	**≥2**	**≥3**	**≥4**	**≥5**	**≥2**	**≥3**	**≥4**	**≥5**	**≥2**	**≥3**	**≥4**	**≥5**
Overall No. (%) of false positive strains	11 (47.8)	8 (34.8)	4 (17.4)	3 (13.0)	13 (56.5)	12 (52.2)	8 (34.8)	4 (17.4)	9 (39.1)	6 (26.1)	2 (8.7)	- (0.0)	13 (56.5)	10 (43.5)	6 (26.1)	2 (8.7)
- *E. coli* [a]	9 (90.0)	8 (80.0)	4 (40.0)	3 (30.0)	9 (90.0)	8 (80.0)	7 (70.0)	4 (40.0)	8 (80.0)	6 (60.0)	2 (20.0)	- (0.0)	9 (90.0)	8 (80.0)	6 (60.0)	2 (20.0)
- Other species [b]	2 (15.4)	- (0.0)	- (0.0)	- (0.0)	4 (30.8)	4 (30.8)	1 (7.7)	- (0.0)	1 (7.7)	- (0.0)	- (0.0)	- (0.0)	4 (30.8)	2 (15.4)	- (0.0)	- (0.0)
Overall No. (%) of false negative strains	9 (36.0)	22 (88.0)	25 (100)	25 (100)	2 (8.0)	8 (32.0)	19 (76.0)	24 (96.0)	13 (52.0)	24 (96.0)	25 (100)	25 (100)	2 (8.0)	14 (56.0)	24 (96.0)	24 (96.0)
- *E. coli* [a]	7 (33.3)	19 (90.5)	21 (100)	21 (100)	- (0.0)	5 (23.8)	16 (76.2)	20 (95.2)	12 (57.1)	21 (100)	21 (100)	21 (100)	1 (4.8)	11 (52.4)	21 (100)	21 (100)
- Other species [b]	2 (50.0)	3 (75.0)	4 (100)	4 (100)	2 (50.0)	3 (75.0)	3 (75.0)	4 (100)	1 (25.0)	3 (75.0)	4 (100)	4 (100)	1 (25.0)	3 (75.0)	3 (75.0)	3 (75.0)
Overall Sensitivity (SN), %	64.0	12.0	0.0	0.0	92.0	68.0	24.0	4.0	48.0	4.0	0.0	0.0	92.0	44.0	4.0	4.0
- *E. coli*	66.7	9.5	0.0	0.0	100	76.2	23.8	4.8	42.9	0.0	0.0	0.0	95.2	47.6	0.0	0.0
- Other species	50.0	25.0	0.0	0.0	50.0	25.0	25.0	0.0	75.0	25.0	0.0	0.0	75.0	25.0	25.0	25.0
Overall Specificity (SP), %	52.2	65.2	82.6	87.0	43.5	47.8	65.2	82.6	60.9	73.9	91.3	100.0	43.5	56.5	73.9	91.3
- *E. coli*	10.0	20.0	60.0	70.0	10.0	20.0	30.0	60.0	20.0	40.0	80.0	100	10.0	20.0	40.0	80.0
- Other species	84.6	100	100	100	69.2	69.2	92.3	100	92.3	100	100	100	69.2	84.6	100	100

Legend. Data highlighted in grey indicate the key interpretative cut-off criteria used to show and discuss the results of the present work. COL, colistin; COL-R, colistin-resistant; COL-S, colistin-susceptible

[a] Overall, 31 strains of which 21 *mcr*-positive. Specifically, one COL-S (*E. coli* ATCC35218) and 30 COL-R strains of which 21 *mcr-1/-2*-positive. See also Table S1 for more details about strains tested and single test results, respectively.

[b] Overall, 17 strains of which 4 *mcr*-positive. Specifically, 3 COL-S (*K. pneumoniae* ATCC700603, *Salmonella enterica*, and *Serratia fonticola*) and 14 COL-R strains (*K. pneumoniae*, n=5 of which 3 *mcr-1*-positive; *K. oxytoca*, n=1; *Salmonella* spp., n=2 of which 1 *mcr-4*-positive; *E. cloacae*, n=1; *H. alvei*, n=1; *M. morganii*, n=1; *Proteus* spp., n=2; *P. stuartii*, n=1). See also Table S1 for more details about strains tested and single test results, respectively.

[c] Adding 10 µl of EDTA at concentration of 100 mM [19].

[d] Adding 10 µl of EDTA at concentration of 200 mM

HIGHLIGHTS

- In several setting (e.g., low income countries) phenotypic tests to detect mcr producers are still essential
- Recently, it has been suggested that an EDTA-based disk-combination test is reliable for this purpose
- We evaluated the ability of several EDTA-based combined-disk tests to detect 25 mcr producers among 48 *Enterobacteriaceae*
- All tests resulted unreliable with sensitivity ≤68% and specificity ≤74%